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# *In vitro* organogenesis of *Eucalyptus grandis*: effects of boron and calcium

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**ABSTRACT.** The *in vitro* organogenesis of woody species plays an essential role in the improvement of forest products by providing saplings with high commercial value. Furthermore, mineral nutrition plays an important role in the induction of organogenic responses. The objective of this study was to evaluate the effects of boron and calcium in the organogenesis of nodal segments from seedlings of *Eucalyptus grandis* growing under *in vitro* conditions. The concentration of boron and calcium in MS medium was modified to induce organogenic responses in 45-day-old nodal segments used as explants. After 60 days, the fresh weight, dry weight, ratio of fresh and dry weight, relative water content and relative matter content accumulated by the explants were evaluated. The concentrations of boron and calcium in the culture medium influenced the *in vitro* organogenic control of *Eucalyptus grandis*. Reduced combinations of boron and calcium induced callus formation and dry matter accumulation in the explants. A boron concentration of 100% (1.10 mg $L^{-1}$) combined with 100% (119.950 mg $L^{-1}$) and 200% (239.900 mg $L^{-1}$) of calcium, and 200% (2.20 mg $L^{-1}$) of boron combined with 100% (119.950 mg $L^{-1}$) of calcium allowed the induction of well-developed buds, which can be used for the regeneration of micro-plants.

**Keywords:** bud induction, callogenesis, tissue culture, mineral nutrition.

## Efeitos do boro e cálcio na organogênese *in vitro* de *Eucalyptus grandis*

**RESUMO.** A organogênese *in vitro* de espécies lenhosas caracteriza-se como fator fundamental para a obtenção de mudas com interesse comercial, gerando aplicações práticas na área do melhoramento florestal. Adicionalmente, a nutrição mineral desempenha papel importante nas respostas de indução organogênica. Objetivou-se avaliar o efeito do boro e cálcio na organogênese de segmentos nodais oriundos de plântulas de *Eucalyptus grandis* germinadas *in vitro*. Segmentos nodais com 45 dias após a germinação foram utilizados como explantes. Os explantes foram submetidos a combinações de boro e cálcio em meio de cultura MS, visando induzir diferentes respostas organogênicas. Ao final de 60 dias de cultivo *in vitro* foram avaliados a matéria fresca, a matéria seca, relação entre matéria fresca e matéria seca, conteúdo relativo de água e conteúdo relativo de matéria acumulada pelos explantes. As concentrações de boro e cálcio no meio de cultura influenciaram o controle organogênico *in vitro* de *Eucalyptus grandis*. Combinações reduzidas de boro e cálcio induziram a formação de calo e acúmulo de matéria nos explantes. A concentração de 100% (1,10 mg $L^{-1}$) de boro combinada com 100% (119,950 mg $L^{-1}$) e 200% (239,900 mg $L^{-1}$) de cálcio e, 200% (2,20 mg $L^{-1}$) de boro combinado com 100% (119,950 mg $L^{-1}$) de cálcio favoreceram a indução de gemas com desenvolvimento normal, podendo ser utilizadas para a regeneração de microplântulas.

**Palavras-chave:** indução de gemas, calogênese, cultura de tecido, nutrição mineral.

## Introduction

The improvement of woody species for planted forest is important to the development of the agricultural sector (ALTMAN, 2003; GROSSNICKLE; SUTTON, 1999; NEHRA et al., 2005), especially for the cultivation of species of high economic interest, such as *Eucalyptus grandis* (MAURICE et al., 2010; STAPE et al., 2001). The development of biotechnological methods enabled the achievement of higher productivity in smaller areas through the selection of phenotypes with characteristics of interest and high adaptability in plantation systems (DUTRA et al., 2009; NEHRA et al., 2005).

The vegetative propagation of *Eucalyptus* species, obtained by cutting (ALMEIDA et al., 2007; CORRÊA

et al., 2005; DÍAZ et al., 2009; HOAD; LEAKEY, 1995), mini-cutting (BORGES et al., 2011; BRONDANI et al., 2010; GOULART et al., 2010; SCHWAMBACH et al., 2008; WENDLING; XAVIER, 2005) or micro-cutting (NAKHOODA et al., 2011; NOURISSIER; MONTEUUIS, 2008) improved the uniformity of the forests, their productivity and the quality of forest products (CHEN et al., 2001; KELLISON, 2007), thereby fostering the progress of the forestry sector in recent decades. Furthermore, numerous techniques of *in vitro* cultivation have been developed to provide greater rooting, thus promoting the rejuvenation of selected plants with superior characteristics (DUTRA et al., 2009; NEHRA et al., 2005).

Another advantage of *in vitro* culture is the greater environmental control of growth conditions (e.g., light, temperature, photoperiod, nutrition, and water availability). These factors are primarily important in studies related to plant nutrition. Moreover, organogenic control is made possible by tissue culture (ALVES et al., 2004; CHRISTIANSON; WARNICK, 1988; GEORGE et al., 2008) and has applications to genetic engineering, as in the development of transgenic plants (ALCANTARA et al., 2011; SILVA et al., 2011). These advances allow the generation of plants with greater adaptive potential under biotic and/or abiotic conditions.

For the successful *in vitro* cultivation of a particular forest woody species, full knowledge of mineral nutrition is necessary if nutritional factors interfere significantly with the organogenic response (HARTMANN et al., 2011; GEORGE et al., 2008). Among the essential elements involved in organogenic control, boron and calcium are emphasized because they are considered essential for plant growth and development (EPSTEIN; BLOOM, 2004; TREVIZAM et al., 2011). Boron is involved in complexes which are components of the cell wall (e.g., mannitol and polymannuronic acid), influences enzyme activity (e.g., polyphenol oxidase and peroxidase) and is also involved in cell division and elongation (DORDAS; BROWN, 2005; MATOH; KOBAYASHI, 1998; TANAKA; FUJIWARA, 2008). Calcium interferes with the integrity of the cell wall, especially in meristematic growth regions, and it is considered one of the most important signaling agents in metabolic pathways (DAYOD et al., 2010; MATOH; KOBAYASHI, 1998; PANDEY, 2008).

Plant regeneration from *in vitro* organogenesis is only possible due to the induction of stimuli for certain metabolic pathways that will trigger changes in the pattern of cell growth and development (PAPP; PLATH, 2011; SMET; BEECKMAN, 2011; ALMEIDA et al., 2012) which are primarily controlled by the interaction between plant growth regulators (e.g., auxins and cytokinins) (ARYA et al., 2009; BENNETT et al., 1994) and mineral nutrition (DUTRA et al., 2009; HUNG; TRUEMAN, 2010; TREVIZAM et al., 2011). Nevertheless, the organogenic response may also vary according to genotype (ALVES et al., 2004) and the environmental conditions associated with growth (GEORGE et al., 2008). Information about the biochemical stimuli associated with nutritional factors is the key to the success of *in vitro* cultivation. This method of cultivation can be applied to the multiplication of selected individuals and the genetic engineering of woody plants.

Based on these observations, we aimed to evaluate the effect of boron and calcium in the *in vitro* organogenesis of nodal segments of *Eucalyptus grandis*.

## Material and methods

### General characterization of the experiment

Seeds of *Eucalyptus grandis* W. Hill. Ex Maiden were collected from a seed orchard (SO) located at the Anhembi, São Paulo State, Brazil. The SO belongs to the Institute of Research and Forestry Studies (Instituto de Pesquisa e Estudos Florestais - IPEF). The cultivar was identified as LCFA001/A11A21 and corresponded to the lot AN0166N01 collection of the 2007 harvest (ALCÂNTARA et al., 2011).

### Asepsis and standardization of the explants

The seeds were washed with running water for 5 min. and then washed further with deionized and autoclaved water. They were then immersed in 70% water-alcohol solution for 30 s, washed with deionized and autoclaved water and immersed in a solution of sodium hypochlorite (NaOCl) with 2.5% of active chlorine for 20 min., followed by washing with deionized and autoclaved water. They were subsequently immersed in a 1% solution of fungicide (*Benomyl*®) for 20 min. and finally washed three times with deionized and autoclaved water (ALCÂNTARA et al., 2011). The seeds were germinated in a recipient glass (6.5 x 7.0 cm) containing 40 mL of MS medium (MURASHIGE; SKOOG, 1962) free of plant growth regulator. At 45 days after *in vitro* germination (Figure 1A), the nodal segments (explants) were collected from the middle third of the seedlings (Figure 1B and C). The leaves and apex were completely removed from the explants.

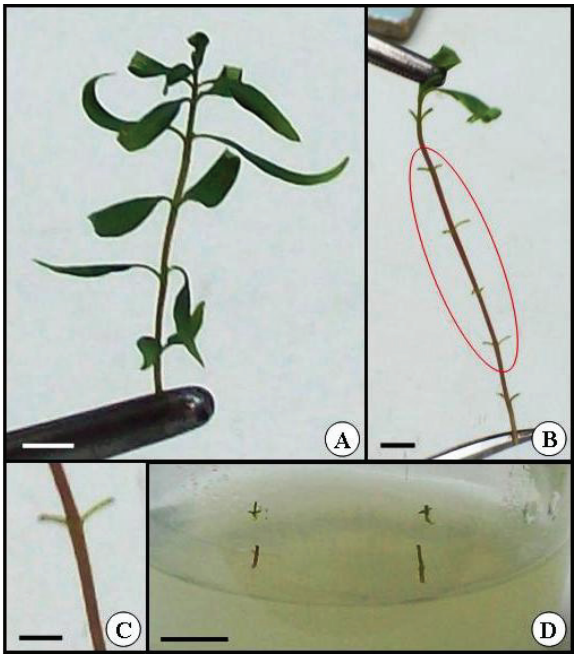


**Figure 1.** Standardization of explants of *Eucalyptus* arranged in the culture medium, bar = 1 cm. *grandis*. (A) shoot of seedling germinated *in vitro* at 45 days, bar = 0.5 cm; (B) detail of the internodes considered for the experiment and removal of leaves, bar = 0.5 cm; (C) nodal segments used as explants, bar = 0.25 cm; (D) nodal segments

### Experimental unit and treatments

The experimental unit was a recipient glass (6.5 x 7.0 cm) containing 40 mL of culture medium and four explants, which were arranged randomly on the surface of the culture explants were submitted to different combinations of boron (B) and calcium (Ca) concentrations (Figure 1D). The concentrations of these elements in the MS culture medium (100%) were used as the standard medium (Table 1).

**Table 1.** Treatments with boron (B) and calcium (Ca) for *in vitro* culture of *Eucalyptus grandis*.

| Treatment | Boron[(1)] | | Calcium[(1)] | |
|---|---|---|---|---|
| | (%) | (mg $L^{-1}$) | (%) | (mg $L^{-1}$) |
| T01 | 0 | 0.00 | 0 | 0.000 |
| T02 | 50 | 0.55 | 0 | 0.000 |
| T03 | 100 | 1.10 | 0 | 0.000 |
| T04 | 200 | 2.20 | 0 | 0.000 |
| T05 | 0 | 0.00 | 50 | 59.975 |
| T06 | 50 | 0.55 | 50 | 59.975 |
| T07 | 100 | 1.10 | 50 | 59.975 |
| T08 | 200 | 2.20 | 50 | 59.975 |
| T09 | 0 | 0.00 | 100 | 119.950 |
| T10 | 50 | 0.55 | 100 | 119.950 |
| T11 | 100 | 1.10 | 100 | 119.950 |
| T12 | 200 | 2.20 | 100 | 119.950 |
| T13 | 0 | 0.00 | 200 | 239.900 |
| T14 | 50 | 0.55 | 200 | 239.900 |
| T15 | 100 | 1.10 | 200 | 239.900 |
| T16 | 200 | 2.20 | 200 | 239.900 |

[(1)]Concentration of nutrients for the composition of culture medium (100% refers to the standard concentration of MS culture medium (MURASHIGE; SKOOG, 1962), B = 1.10 mg $L^{-1}$ and Ca = 119.95 mg $L^{-1}$).

The culture medium was prepared with the following salts: $NH_4NO_3$, $KNO_3$, $CaCl_2.2H_2O$, $KH_2PO_4$, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $H_3BO_3$, KI, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$ and $CoCl_2.6H_2O$.

### Preparation of culture medium and cultivation conditions

The MS medium (MURASHIGE; SKOOG, 1962) was supplemented with 30 g $L^{-1}$ sucrose and 9 g $L^{-1}$ agar. The pH was adjusted to 5.8 with KOH (1M) and/or HCl (1M) before adding the agar. After this step, the culture medium was autoclaved for 20 min. at 121°C (≈1.0 kgf $cm^{-2}$). The culture medium was supplemented with 0.50 mg $L^{-1}$ BAP (6-benzylaminopurine) and 0.05 mg $L^{-1}$ NAA (α-naphthalene acetic acid). The experimental units were placed in a growth chamber at a temperature of 27°C (± 2°C), a photoperiod of 12 hours and a light intensity of 40 $\mu$mol $m^{-2}$ $s^{-1}$. At 30 days, a subculture was established with the same treatments.

### Data collection

Fresh matter (FM), dry matter (DM), the ratio of fresh and dry matter (RFDM), the relative water content (RWC) and the relative matter content (RMC) of explants were evaluated at 60 days of *in vitro* cultivation (Table 2). Visual analysis was used to identify changes in the pattern of morphogenetic development and coloring (deficiency or toxicity) of the explants. The dry matter per explant was determined from the weighing of the samples dried in an oven at 60°C until a constant weight was attained. The fresh and dry matter per explant were determined using an analytical balance (0.001 g).

**Table 2.** Formulas used to determine the ratio of fresh and dry matter (RFDM), the relative water content (RWC) and the relative matter content (RMC) of explants of *Eucalyptus grandis* in relation to the B and Ca concentrations at 60 days of *in vitro* cultivation.

| Variable | Formula | Unit |
|---|---|---|
| Ratio of fresh and dry matter (RFDM) | $RFDM = \frac{FM}{DM}$ | – |
| Relative water content (RWC) | $RWC = \left(\frac{FM - DM}{FM}\right) \cdot 100$ | % |
| Relative matter content (RMC) | $RMC = \left(\frac{DM}{FM}\right) \cdot 100$ | % |

Note: FM = fresh matter content per explant, DM = dry matter content per explant.

### Experimental design and statistical analysis

The experiment was conducted with a randomized design in a 4 x 4 factorial arrangement that tested 16 combinations of B and Ca (Table 1), five replications and four explants per replication. The data were subjected to a Hartley test ($p < 0.05$) to verify the

homogeneity of variance between treatments. We also applied an analysis of variance to the data (ANOVA, $p < 0.01$ and $p < 0.05$). Based on their significance, the means were subjected to a regression analysis ($p < 0.05$). We used SOC software (EMBRAPA, 1990) to perform the statistical procedures.

## Results and discussion

The growth characteristics of the explants of *Eucalyptus grandis* measured at 60 days under *in vitro* conditions varied significantly according to the levels of boron and calcium and resulted in different organogenic responses. The ANOVA revealed interactions between the concentrations of boron and calcium for fresh matter ($p < 0.01$), dry matter ($p < 0.05$), the ratio of fresh and dry matter ($p < 0.01$), the relative water content ($p < 0.01$) and the relative matter content ($p < 0.01$) (Table 3).

With calcium absent from the culture medium, the fresh matter per explant increased with increasing boron concentration up to the highest concentration (200% = 2.20 mg $L^{-1}$). With increasing amounts of boron and calcium in combination, the amount of fresh matter per explant decreased (Figure 2A). A similar effect occurred for dry matter per explant. With calcium absent from the culture medium, the dry matter per explant increased with increasing boron concentration up to the highest concentration (200% = 2.20 mg $L^{-1}$). With increasing amounts of boron and calcium in combination, the amount of dry matter per explant decreased (Figure 2B). It is probable that this effect is associated with the greater induction of callus formation in the calcium-free medium (the presence of calluses in all explants) that was preceded by matter accumulation. The induction of shoots and leaves with the increasing amounts of boron and calcium in combination in the culture medium promoted decreases in the fresh and dry matter per explant of *Eucalyptus grandis*.

The ratio of fresh and dry matter per explant reached its critical point at 78.90% (0.86 mg $L^{-1}$) boron and 24.08% (28.88 mg $L^{-1}$) calcium, a value of 10.30 (Figure 2C). The relative water content per explant increased up to the greatest amounts of boron and calcium, i.e., the explants tended toaccumulate more water as the availability of boron and calcium in the culture medium increased (Figure 2D). The relative matter content accumulated per explant was higher in the absence of calcium in combination with 100% (1.10 mg $L^{-1}$) boron in the culture medium (Figure 2E).

The combinations of 0 and 50% (59.975 mg $L^{-1}$) calcium in the absence of boron favored the induction of callus in explants of *Eucalyptus grandis* at 60 days of *in vitro* cultivation. This result showed that if these elements were present in low concentrations in the culture medium or were absent, the induction of undifferentiated cells (i.e., callus formation) occurred. The callus exhibited a gelatinous appearance and a dark color. Callus formation was followed by low-grade induction of buds (Figure 3A and E). In the absence of boron in the culture medium and with 100% (119.950 mg $L^{-1}$) and 200% (239.900 mg $L^{-1}$) calcium, the induction of buds occurred, but the buds formed a rosette and were chlorotic, both symptoms of nutritional deficiency (Figure 3I and M). In the combinations of 50% (0.55 mg $L^{-1}$) boron with 0 and 50% (59.975 mg $L^{-1}$) calcium in the culture medium, buds appeared. These buds were chlorotic with reddish pigmentation, indicating that the development of the explants was abnormal (Figure 3B and F). The combination of 50% (0.55 mg $L^{-1}$) boron with 100% (119.950 mg $L^{-1}$) and 200% (239.900 mg $L^{-1}$) calcium resulted in the normal induction of buds. However, these buds were small (Figure 3J and N). Results similar were observed for the combination of 100% (1.10 mg $L^{-1}$) boron with 0 and 50% (59.975 mg $L^{-1}$) calcium and for the combination of 200% (2.20 mg $L^{-1}$) boron with 0 and 50% (59.975 mg $L^{-1}$) calcium (Figure 3C, D, G and H). The best induction of buds presenting a vigorous appearance, normal development and an absence of chlorosis occurred in combinations of 100% (1.10 mg $L^{-1}$) boron with 100% (119.950 mg $L^{-1}$) and 200% (239.900 mg $L^{-1}$) calcium and 200% (2.20 mg $L^{-1}$) boron combined with 100% (119.950 mg $L^{-1}$) calcium (Figure 3K, L and O). The combination of 200% (2.20 mg $L^{-1}$) boron with 200% (239.900 mg $L^{-1}$) calcium resulted in the induction of chlorotic buds with small leaves, indicating toxicity (Figure 3P).

The control of *in vitro* organogenesis in plants promoted the development of biotechnology, creating applications in several areas of knowledge. In a practical sense, progress in the agricultural sector was significant, both in terms of the quality and quantity of the product generated (ALTMAN, 2003; DUTRA et al., 2009; MAURICE et al., 2010; NEHRA et al., 2005; STAPE et al., 2001). Advances in biotechnology continue to be developed. New discoveries are made each year, primarily through the improvement of analytical techniques.

**Table 3.** Analysis of variance (ANOVA) for fresh matter (FM), dry matter (DM), the ratio of fresh and dry matter (RFDM), the relative water content (RWC) and the relative matter content (RMC) of explants of *Eucalyptus grandis* in relation to the concentrations of boron and calcium at 60 days of *in vitro* cultivation.

| Sources of Variation | DF | Mean Squares | | | | |
|---|---|---|---|---|---|---|
| | | FM[(1)] | DM[(1)] | RFDM[(1)] | RWC[(1)] | RMC[(1)] |
| | | (g explant$^{-1}$) | (g explant$^{-1}$) | – | (% explant$^{-1}$) | (% explant$^{-1}$) |
| Boron (B) | 3 | 2.6026$^{**}$ | 1.7919$^{**}$ | 0.1687$^{*}$ | 0.0030$^{**}$ | 0.2993$^{**}$ |
| Calcium (Ca) | 3 | 0.8567$^{ns}$ | 0.9204$^{*}$ | 0.1342$^{ns}$ | 0.0012$^{*}$ | 0.0580$^{ns}$ |
| B*Ca | 9 | 1.3022$^{**}$ | 0.9285$^{*}$ | 0.1598$^{**}$ | 0.0015$^{**}$ | 0.2211$^{**}$ |
| Residual | 64 | 0.3275 | 0.3122 | 0.0537 | 0.0004 | 0.0592 |
| Average | – | 0.903 | 0.073 | 11.72 | 91.08 | 8.91 |
| CV (%) | – | 28.30 | 31.02 | 20.41 | 20.04 | 20.91 |

$^{ns}$ value not significant at 5% probability by F test. $^{*}$ and $^{**}$ values significant at 5 and 1% probability, respectively, by F test. $^{(1)}$ Data transformed by log($n$) at 5% probability by Hartley test. $n$ = sampled data. CV = experimental coefficient of variation, DF = degrees of freedom.

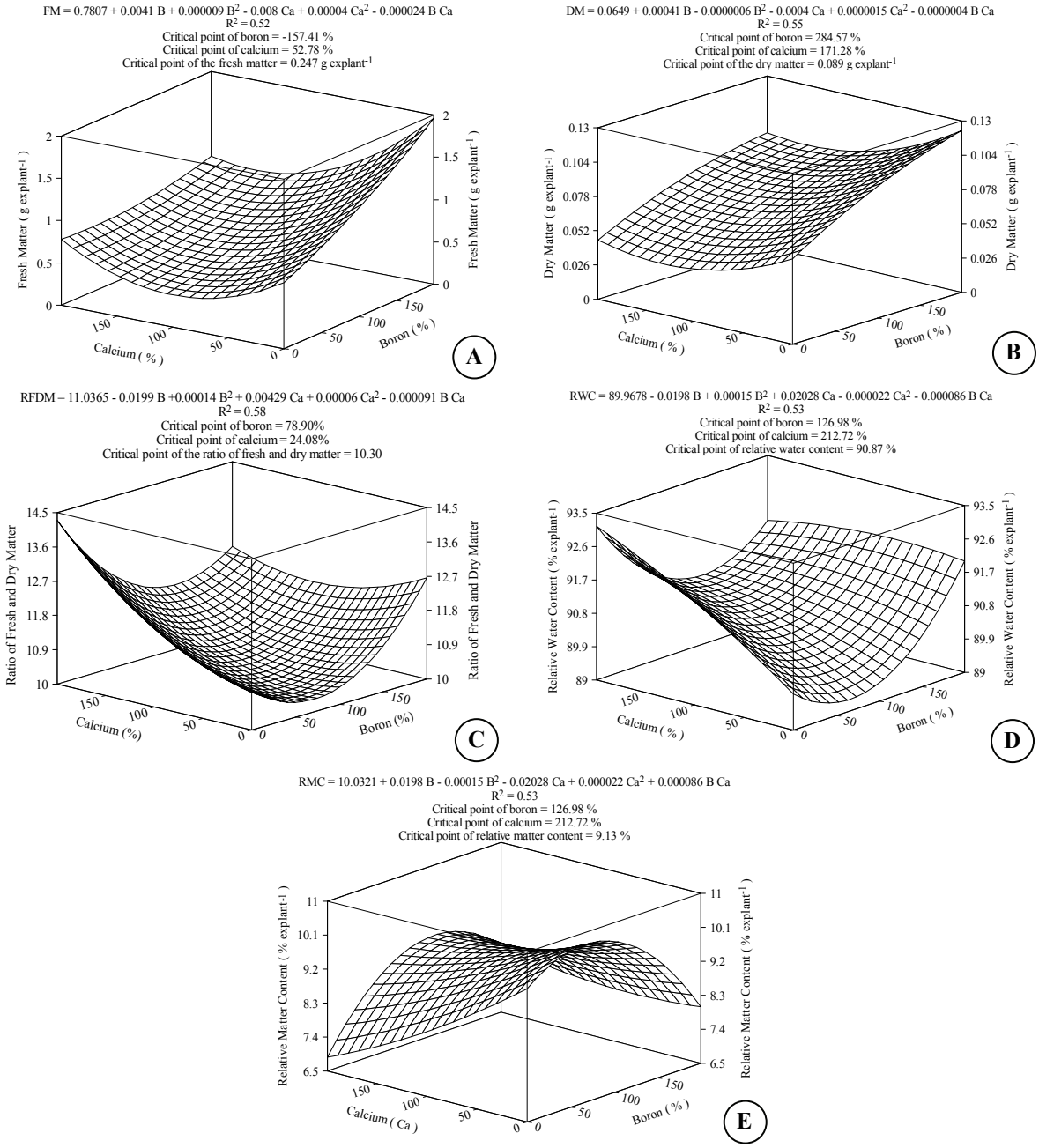


**Figure 2.** Growth characteristics of explants of *Eucalyptus grandis* in response to treatments with boron and calcium at 60 days of *in vitro* cultivation. (A) fresh matter - FM; (B) dry matter - DM; (C) ratio of fresh and dry matter - RFDM; (E) relative water content - RWC; (F) relative matter content - RMC.

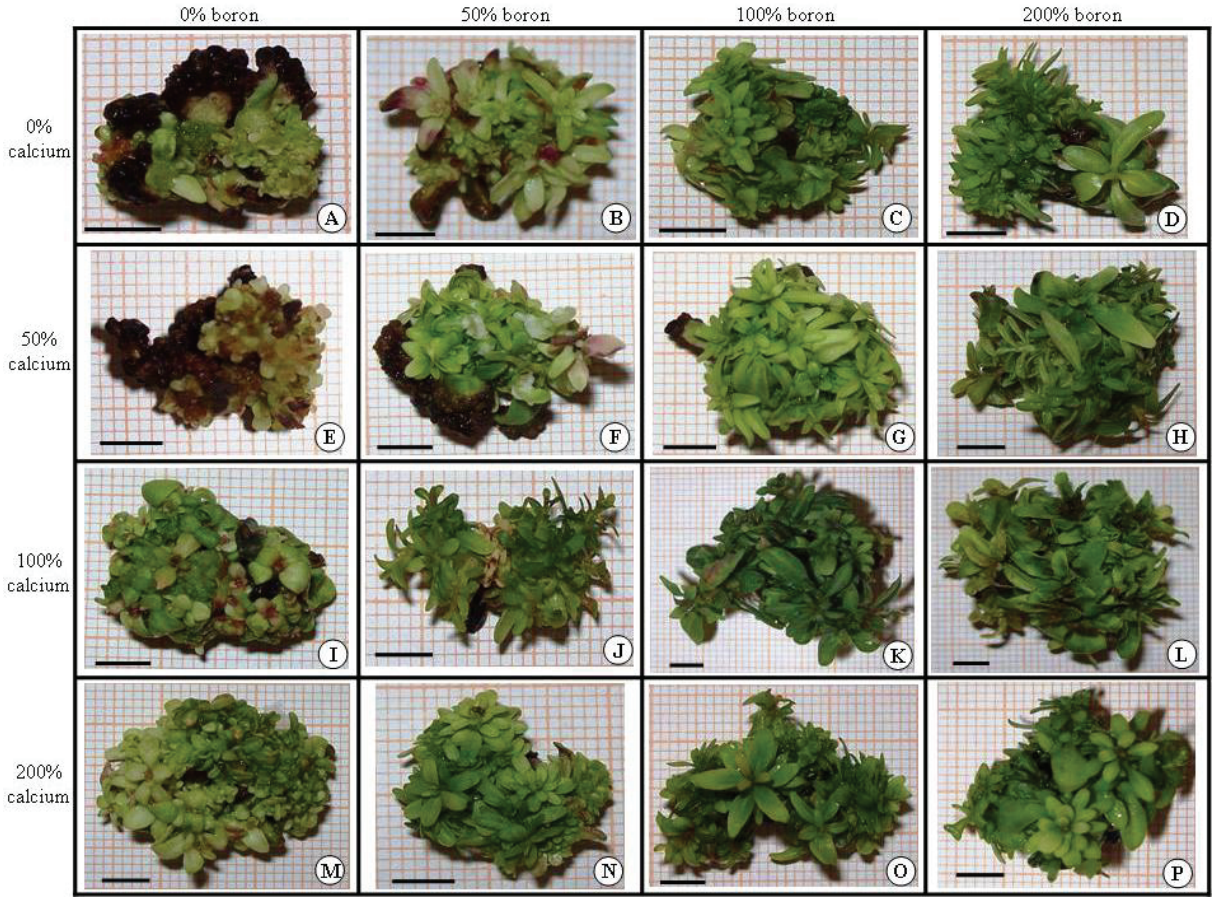


**Figure 3.** Organogenesis of buds from nodal segments of seedlings of *Eucalyptus grandis* germinated *in vitro* at 60 days of culture. (A) free culture medium of boron and calcium; (B) culture medium containing 50% boron; (C) culture medium containing 100% boron; (D) culture medium containing 200% boron; (E) culture medium containing 50% calcium; (F) culture medium containing 50% boron and 50% calcium; (G) culture medium containing 100% boron and 50% calcium; (H) culture medium containing 200% boron and 50% calcium; (I) culture medium containing 100% calcium; (J) culture medium containing 50% boron and 100% calcium; (K) culture medium containing 100% boron and 100% calcium; (L) culture medium containing 200% boron and 100% calcium; (M) culture medium containing 200% calcium; (N) culture medium containing 50% boron and 200% calcium; (O) culture medium containing 100% boron and 200% calcium; (P) culture medium containing 200% boron and 200% calcium. Note: 100% refers to the standard concentration of MS culture medium (MURASHIGE; SKOOG, 1962), B = 1.10 mg $L^{-1}$ and Ca = 119.95 mg $L^{-1}$ (Table 1). Bar = 5 mm.

Progress in this area allowed the generation of additional knowledge about physiological factors, such as the nutritional aspects of the organogenesis of plant tissues *in vitro*. B and Ca influence several metabolic pathways (DAYOD et al., 2010; DORDAS; BROWN, 2005; MATOH; KOBAYASHI, 1998; PANDEY, 2008; TANAKA; FUJIWARA, 2008) that act on growth and tissue development. Christianson and Warnick (1988) classified the stages of organogenesis as dedifferentiation, competence acquisition, induction, determination, differentiation and organ formation. Among these organogenic responses, the induction of callus and buds are recorded in several reports, but the responses are highly dependent on genotype and culture conditions *in vitro*, effects that were observed in this study with *Eucalyptus grandis*.

Increasing concentrations of B and Ca in the culture medium influenced the *in vitro* organogenic response in explants of *Eucalyptus grandis*, and the simultaneous omission of these elements resulted in the induction of callus (Figure 3A). A similar effect was reported by Trevizam et al. (2011), who reported that the simultaneous omission of B and Ca in callus culture of *Eucalyptus urophylla* inhibited root formation, caused the disintegration of calluses, and favored the formation of friable and globular structures and the occurrence of anthocyanin. Furthermore, high concentrations of calcium induced roots in the callus. Boron deficiency in the culture medium can cause the accumulation of phenols, necrosis, callus and abnormal induction of adventitious buds (DORDAS; BROWN, 2005; MATOH; KOBAYASHI, 1998; TANAKA;

FUJIWARA, 2008; TREVIZAM et al., 2011), characteristics that were observed in this study with *Eucalyptus grandis* (Figure 3A, B, E and F).

The study's findings regarding the regeneration of buds showed that explants from the nodal segments cultivated *in vitro* exhibited a high morphogenetic potential for the multiplication of buds. This effect is in agreement with the observations of George et al. (2008) that in many forest species, explants from hypocotyl, epicotyl and young tissues of plants grown *in vitro* have a high morphogenetic potential. This high potential arises primarily because these tissues have a high potential for exhibiting juvenility, associated with a gradient of endogenous growth regulators. The best induction of buds in the explants of *Eucalyptus grandis* was associated with the supplementation of 0.50 mg $L^{-1}$ BAP and 0.05 mg $L^{-1}$ NAA in culture medium and was also very dependent on the concentrations of B and Ca, i.e., the effects of the plant growth regulators controlling organogenesis were also dependent on variations in the nutrient culture medium *in vitro*. This effect is consistent with the observations of Altman (2003), Alves et al. (2004), Brondani et al. (2009), Dutra et al. (2009), Huang et al. (2010), Hung and Trueman (2010), Alcântara et al. (2011), Brondani et al. (2011), Borges et al. (2011), Nakhooda et al. (2011), and Trevizam et al. (2011), which highlight the importance of nutrition in the *in vitro* cultivation of plants.

The accumulation of fresh and dry matter is also associated with nutrition (HUNG; TRUEMAN, 2010), such as the availability of B and Ca in the culture medium (GEORGE et al., 2008). These previous findings were confirmed by the results of this study. Under *ex vitro* conditions, boron deficiency causes growth inhibition of *Eucalyptus grandis* seedlings, especially in the regions of the meristematic shoots, thereby impairing the initiation of apical and lateral buds (BARRETTO et al., 2007). The effect of B and Ca in the culture of callus is demonstrated by the finding of Trevizam et al. (2011) that Ca and B act synergistically *in vitro* in organogenic processes in *Eucalyptus urophylla*.

The relative water content in the explants showed significant variation, primarily related to the type of cell structure induced, whereas the presence of callus was preceded by an increased accumulation of matter and less water retention in the tissues. Water loss from the cells to the culture medium or the processes of autolysis characterizes the protective response of the plant under conditions of stress (TREVIZAM et al., 2011). Furthermore, the exhaustion of minerals in the culture medium may have induced the callus to develop a chronic deficiency of one or more nutrients essential to cell metabolism. This deficiency could have influenced the growth and development of the explants and affected factors related to organogenesis (DAYOD et al., 2010; GEORGE et al., 2008; TANAKA; FUJIWARA, 2008).

In general terms, different organogenic responses (callus induction and/or buds) in the nodal segments of *Eucalyptus grandis* resulted from the interactions between the nutritional properties of B and Ca. These results indicated the influence of these elements on the organogenic process of *in vitro* cultivation. Organogenic control can be induced by hormonal changes (primarily by the action of auxins and cytokinins) but is also dependent on the nutritional factors that influence the metabolic reactions. We observe that in addition to genetic factors, the culture conditions *in vitro* (such as nutrients and plant growth regulators) are responsible for the production of diverse responses, as indicated by several reports on the *in vitro* culture of plants. Therefore, there is no single best protocol, but there is a need for constant adjustment of the culture medium to obtain optimum growth and organogenic development of the genotype of interest.

With adequate knowledge of the factors that govern such events, progress can be made in the regeneration of plants from just one responsive organ that presents cellular competence. New advances in genetic engineering are expected, especially in the cultivation of new varieties of transgenic plants, and preliminary studies of *in vitro* organogenesis from different tissue types are complementary to the improvement of plant tissue culture techniques.

## Conclusion

The concentrations of boron and calcium in the culture medium influenced the control of *in vitro* organogenesis in *Eucalyptus grandis*.

Low levels of boron and calcium in combination induced callus formation and the accumulation of matter in the explants.

The concentration of 100% (1.10 mg $L^{-1}$) boron combined with 100% (119.950 mg $L^{-1}$) and 200% (239.900 mg $L^{-1}$) calcium and the concentration of 200% (2.20 mg $L^{-1}$) boron combined with 100% (119.950 mg $L^{-1}$) calcium favored the induction of buds with normal development. These buds can be used for the regeneration of micro-plants.

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